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Purification, Stability, and Immunogenicity Analyses of Five Bluetongue Virus Proteins for Use in Development of a Subunit Vaccine That Allows Differentiation of Infected from Vaccinated Animals

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Bluetongue virus (BTV) causes bluetongue disease, a vector-borne disease of ruminants. The recent northerly spread of BTV serotype 8 in Europe resulted in outbreaks characterized by clinical signs in cattle, including unusual teratogenic effects. Vaccination has been shown to be crucial for controlling the spread of vector-borne diseases such as BTV. With the aim of developing a novel subunit vaccine targeting BTV-8 that allows differentiation of infected from vaccinated animals, five His-tagged recombinant proteins, VP2 and VP5 of BTV-8 and NS1, NS2, and NS3 of BTV-2, were expressed in baculovirus or *Escherichia coli* expression systems for further study. Optimized purification protocols were determined for VP2, NS1, NS2, and NS3, which remained stable for detection for at least 560 to 610 days of storage at +4°C or −80°C, and Western blotting using sera from vaccinated or experimentally infected cattle indicated that VP2 and NS2 were recognized by BTV-specific antibodies. To characterize murine immune responses to the four proteins, mice were subcutaneously immunized twice at a 4-week interval with one of three protein combinations plus immunostimulating complex ISCOM-Matrix adjuvant or with ISCOM-Matrix alone ($n = 6$ per group). Significantly higher serum IgG antibody titers specific for VP2 and NS2 were detected in immunized mice than were detected in controls. VP2, NS1, and NS2 but not NS3 induced specific lymphocyte proliferative responses upon restimulation of spleen cells from immunized mice. The data suggest that these recombinant purified proteins, VP2, NS1, and NS2, could be an important part of a novel vaccine design against BTV-8.

Bluetongue (BT) disease is a transboundary disease of ruminants caused by BT virus (BTV), a double-stranded RNA virus of the family *Reoviridae*. BTV is primarily transmitted by biting midges (*Culicoides* species) and like other vector-borne viruses is difficult to control using conventional biosecurity measures (1, 2). Therefore, vaccination campaigns are important tools to prevent virus spread and clinical BT disease (3). In Europe, modified live virus vaccines (MLVs) and inactivated vaccines have helped to limit recent outbreaks of BTV (3), including BTV-8, which is characterized by clinical signs in cattle (4) and a northerly spread (3). However, the use of MLVs is no longer recommended due to safety-related drawbacks (5–9). Inactivated vaccines, while safer, cost more to produce (10) and like MLVs can complicate epidemiological surveillance of BTV infection and vaccine efficacy (11). Therefore, there is a need for novel vaccines that allow the differentiation of infected from vaccinated animals (DIVA) and that can quickly be adapted to new or multiple BTV serotypes (12).

Next-generation BTV vaccines aim to fulfill these requirements while also providing the safety and efficacy offered by current vaccines. Experimental vaccines, including disabled infectious single-cycle vaccines, virus-like particles, and subunit vaccines, rely on excluding at least one BTV protein so that detected antibodies against that protein indicate infection rather than vaccination. Thus, they are often protein based using expression systems based on viruses (13–18), bacteria (19), yeast (20), or plants (21). To aid purification and thus reduce safety and regulatory concerns (22, 23), affinity tags can be added to expressed antigens. The resulting challenges to vaccine development are not only choosing antigens but also expression systems and purification methods enabling vaccines to be produced quickly and affordably, have a long shelf life, and induce protective immunity against the target pathogen.

The BTV virion consists of three layers comprised of seven structural proteins (VP1 to VP7) surrounding 10 genome segments that also encode five nonstructural proteins (NS1 to -4 and NS3A). VP2 and VP5 compose the virus's outermost layer. VP2 is the primary target of neutralizing antibody responses induced by BTV infection, and its high variability permits differentiation of the 26 BTV serotypes (8, 24). Individual serotypes do not confer full protection against each other (25–27). Therefore, VP2 is crucial for serotype-specific protection against BT disease, likely through neutralizing antibody induction (17, 28, 29). It has been suggested that VP5 may aid this induction by supporting the VP2

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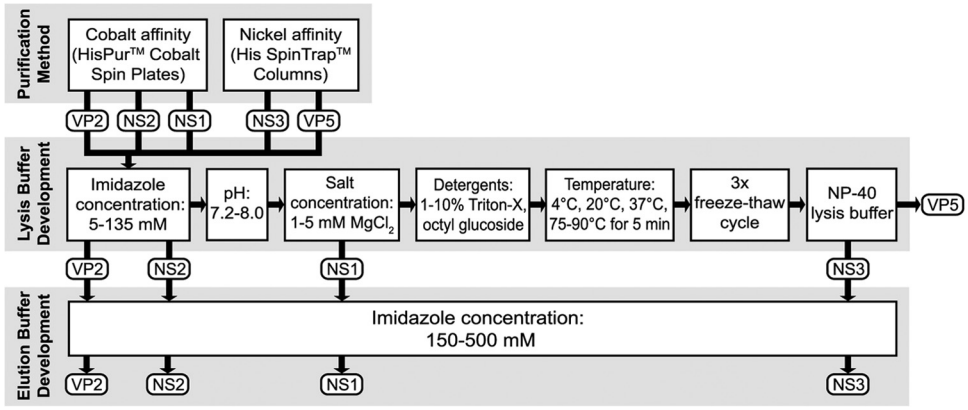

FIG 1 Optimization process of purification protocols for recombinant BTV proteins. Variations in the buffers were tried in sequence as indicated horizontally, until acceptable purity was achieved.

tertiary conformation (17). However, despite identification of epitopes on VP5 that are recognized by serum antibodies from infected ruminants (30, 31), the protein's individual role in inducing protection is not entirely understood.

Within the BTV outer capsid, an inner capsid composed of VP7 surrounds a VP3 layer, which encloses the genome and is attached to transcriptase complexes formed by VP1, VP4, and VP6 (32). Compared to VP2 and VP5, these proteins are more conserved across serotypes. VP7, which is highly immunogenic (33), is widely used in serological diagnosis. Despite evidence that some inner capsid proteins may induce various degrees of immunity (14, 34–37), their specific contributions to protection are not fully elucidated.

In contrast, it is accepted that the nonstructural proteins produce protective cell-mediated immune responses (38, 39). Both NS1 and NS2 have been shown to induce specific humoral or cellular immunity (16, 39–41), while NS3 is understood to induce specific immunological responses but to a lesser degree (40, 42). Notably, studies in experimentally or naturally infected and vaccinated animals (39–41, 43, 44) indicate that these cellular immune responses may protect against other BTV serotypes. This is important considering the numerous BTV outbreaks of different serotypes in Europe over the past 2 decades.

In this study, we aimed to use rational vaccine design and recombinant proteins expressed in production systems available in our laboratories to develop a novel subunit DIVA vaccine against BTV-8 for use in cattle. This technique may be adapted for other serotypes or further developed into a multiserotype vaccine depending on local BTV epidemiology. We expressed the recombinant proteins VP2 and VP5 of BTV-8 and NS1, NS2, and NS3 of BTV-2, optimized purification protocols, and determined the stability of each protein during storage over time. Next, we evaluated the protein-specific immunogenicities in mice by assessing immune responses induced by different multicomponent formulations of purified VP2, NS1, NS2, and NS3 combined with an ISCOM-based adjuvant. Last, we tested whether purified recombinant proteins were recognized by serum antibodies from BTV-8-vaccinated or infected cattle before proceeding to large-animal experiments.

MATERIALS AND METHODS

Recombinant protein expression. The VP2- and VP5-encoding genes of the BTV-8 French strain (isolated in 2006) and NS1-, NS2-, and NS3-encoding genes of the BTV-2 Corsican strain (isolated in 2001) were either inserted into individual "bacmids" using recombination and expressed in Bac-to-Bac baculovirus expression systems (Invitrogen, United Kingdom) following individual infections of *Spodoptera frugiperda* (Sf9) cells (VP2, VP5, NS1, and NS3; molecular mass, 111, 59, 64, and 35 kDa, respectively) or cloned into a pET28 vector and expressed in *Escherichia coli* BL21-AI (Invitrogen, United Kingdom) (NS2; molecular mass, 40 kDa), following the manufacturers' protocols. All proteins were tagged with 6 His residues for later purification using nickel or cobalt affinity. Sf9 cells (Invitrogen, United Kingdom) infected with different recombinant baculoviruses expressing individual recombinant proteins were harvested after 48 to 96 h. Recombinant NS2 expression was induced for 5 h in medium containing 0.1% L-arabinose and 1 M isopropyl-β-D-thiogalactopyranoside (IPTG). Insect cell or bacterial cell suspensions containing recombinant proteins were centrifuged for 10 min at 300 × *g* or at 500 × *g*, respectively, and frozen at −80°C until purified for *in vitro* immunological assays and experimental immunizations or analyzed concerning stability.

Recombinant protein purification. The purification method for each of the recombinant proteins was selected as a choice between His SpinTrap columns (GE Healthcare, United Kingdom) or HisPur Cobalt Spin Plates (Pierce, USA), and the manufacturers' protocols were optimized with regard to lysis and elution buffers, as shown in Fig. 1 and with final parameters listed in Table 1. Besides using optimized buffers, purifications were performed according to the manufacturers' protocols, with two exceptions: (i) lysed sample that flowed through the columns or plate wells was reloaded onto the same column or plate well and incubated a second time with agitation, and centrifugation was repeated before washing; and (ii) the optimized elution buffer was added 4 times per column or plate well, each time with an incubation step of 5 min at +4°C followed by centrifugation at +4°C and 100 × *g* for 1 min (columns) or 500 × *g* for 3 min (plate wells). Optimized lysis and elution buffers for VP5 were not determined, and therefore, this recombinant protein was omitted from further study.

Before vaccine formulation, the purified proteins VP2, NS1, NS2, and NS3 were dialyzed in Slide-a-Lyzer 10,000-molecular-weight-cutoff (MWCO) dialysis cassettes (Pierce, USA) in sterile phosphate-buffered saline (PBS) for 40 h at +4°C (3 changes of 5 liters buffer). Cellulose acetate syringe filters (0.45 μm) were used to sterile filter VP2 and NS2, and all recombinant proteins were stored at −80°C until use.

Recombinant protein stability. VP2, VP5, NS1, NS2, and NS3 were purified by nickel or cobalt affinity using His SpinTrap columns (VP5 and NS3) or HisPur cobalt spin plates (VP2, NS1, and NS2), respectively, according to the manufacturers' protocols, not to the optimized protocols, and stored at +4°C and −80°C as aliquots before and after purification (called "crude" and "purified," respectively). These aliquots were

TABLE 1 Optimized parameters for purification of recombinant His-tagged BTV proteins VP2, VP5, NS1, NS2, and NS3

Protein (serotype), expression system	Lysis buffer[a]	Purification method	Elution buffer[a]
VP2 (BTV-8), baculovirus/Sf9 cells	50 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole	HisPur cobalt spin plates[b] (cobalt affinity)	50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole
VP5 (BTV-8), baculovirus/Sf9 cells	Not applicable	His SpinTrap columns[c] (nickel affinity)	Not applicable
NS1 (BTV-2), baculovirus/Sf9 cells	1 mM $MgCl_2$, 20 mM imidazole, Benzonase nuclease HC,[d] in PBS	HisPur cobalt spin plates (cobalt affinity)	500 mM imidazole in PBS
NS2 (BTV-2), *E. coli* (BL21)	50 mM sodium phosphate, 300 mM sodium chloride, 5 mM imidazole, 100 μg/ml lysozyme[d]	HisPur cobalt spin plates (cobalt affinity)	50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole
NS3 (BTV-2), baculovirus/Sf9 cells	NP-40 lysis buffer[e]	His SpinTrap columns (nickel affinity)	50 mM sodium phosphate, 300 mM sodium chloride, 500 mM imidazole

[a] All buffers contained EDTA-free complete protease inhibitor cocktail tablets (Roche Applied Sciences, United Kingdom).
[b] Pierce, USA.
[c] GE Healthcare, USA.
[d] Sigma Aldrich, USA. HC, high concentration.
[e] National Veterinary Institute (SVA), Sweden.

tested for the presence of proteins detectable by Western blotting as described below, after storage for 0 days, 7 days (1 week), 14 days (2 weeks), and 35 days (5 weeks) at +4°C and −80°C and after storage for 616 days (88 weeks) at +4°C.

Additional aliquots of VP2, NS1, NS2, and NS3 prepared at different stages of optimized purification for experimental animal immunizations (purified, dialyzed, sterile-filtered VP2 and NS2 and purified NS1) and of purified VP2, NS1, and NS2 following optimized purification protocols were stored at −80°C and tested after 210 days (30 weeks) and 560 days (80 weeks), respectively.

Recombinant protein identification and quantification. Coomassie staining of SDS-PAGE gels and Western blots was used to determine the presence and stability of each protein throughout this study. Protein samples were diluted in Laemmli buffer (Bio-Rad Laboratories, USA), heated at 96°C for 5 min, and then run on an SDS-PAGE gel for 40 min at 200 V. For Coomassie staining, the gel was fixed in isopropanol fixing solution for 30 min at room temperature (RT) and then incubated in Coomassie stain for 2 h at RT before destaining in 10% acetic acid. For Western blotting, the gel was transferred to a nitrocellulose membrane for 1 h at 50 V, blocked in 2% (wt/vol) bovine serum albumin (BSA) in PBS for 1 h at RT, and washed 3 times for 5 min in PBS-Tween before incubation for 2 h at RT with primary antibody (mouse anti-histidine tag monoclonal antibodies [MCA1396; AbDSerotec, United Kingdom]). The membranes were washed again in PBS-Tween and incubated for 1 h at RT in secondary antibody (rat anti-mouse heavy chain IgG1-horseradish peroxidase [HRP] [MCA336P; AbDSerotec, United Kingdom]) before an additional washing step and visualization using stable diaminobenzidine (DAB) (Invitrogen, United Kingdom). Where images of Western blots and Coomassie-stained gels are presented in this article, contrast, brightness, and color balance have been adjusted to optimize image quality.

Mass spectrophotometry (MS), performed at the SciLifeLab (Uppsala, Sweden), was used to verify the identity of each recombinant protein using selected bands from SDS-PAGE gels. Briefly, the protein band for VP2 was in-gel digested using trypsin and then resolved in 5 μl of 30% acetonitrile and 1% formic acid and loaded onto an MTP 384 ground steel target using the dried droplet technique and an α-cyano-4-hydroxycinnamate matrix. Peptide mass mapping was performed in the software program MASCOT (Mascot Science, United Kingdom) after mass spectra were recorded in positive mode on an Ultraflex II matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrophotometer (Bruker Daltonics, Bremen, Germany). Protein bands for VP5, NS1, NS2, and NS3 were also in-gel digested with trypsin and then resolved in 15 μl 0.1% formic acid before the peptides for the corresponding protein bands were separated using a reversed-phase C_{18} column and electrosprayed online to an LTQ-Orbitrap Velos Pro ETD mass spectrometer (Thermo Finnigan). Collision-induced dissociation (CID) was applied to perform tandem mass spectrometry (MS/MS), and database searches were performed in MASCOT (Matrix Science, United Kingdom) against proteins in the NCBI Virus database (www.ncbi.nlm.nih.gov/genomes/VIRUSES/viruses.html).

For comparative analyses, the percent purities of proteins were determined using the gel analysis method of the software program ImageJ (45). Protein quantification for immunological assays and experimental immunizations was determined using a Bradford assay read at 595 nm.

Evaluation of immunogenicity in mice. (i) Animals and experimental design. Twenty-four 6- to 12-week-old female BALB/c mice were housed at the Animal House at the Swedish Veterinary Institute (SVA) (Uppsala, Sweden). The mice were divided into 4 groups of 6 mice each and immunized subcutaneously in the back of the neck at day 0 and day 28 with homologous vaccines prepared from purified recombinant VP2, NS1, NS2, and NS3 (with purities of 95%, 51%, 85%, and 77%, respectively) combined in 3 different formulations: (i) 1.5 μg of VP2 and 5 μg of ISCOM-Matrix adjuvant AbISCO-100 (Isconova AB, Sweden) (name, vVP_2); (ii) 1.5 μg NS1, 1.5 μg NS2, 1.225 μg NS3, and 5 μg AbISCO-100 (name, $vNS_{1/2/3}$); (iii) 1.5 μg VP2, 1.5 μg NS1, 1.5 μg NS2, 1.225 μg NS3, and 5 μg AbISCO-100 (name, $vVP_2NS_{1/2/3}$), per dose; or (iv) 5 μg AbISCO-100 in PBS (name, control). A smaller amount of NS3 was used due to difficulties in producing a sufficient quantity of the purified protein. The final volume of each vaccine formulation was adjusted to 200 μl per dose with sterile PBS.

Blood samples were obtained from the tail of each mouse before the first immunization (day 0), before the second immunization (day 28), and 2 weeks after the second immunization (day 41 or day 42). Euthanization was performed on day 41 or day 42 through dislocation of the cervical spine. This experiment was approved by the Ethical Committee of Uppsala, Sweden (C 237/10).

(ii) Detection of protein-specific antibodies. Serum IgG antibodies specific to VP2 of BTV-8 and NS1, NS2, and NS3 of BTV-2 were detected using indirect enzyme-linked immunosorbent assays (ELISAs). Briefly, 96-well ELISA plates (Maxisorp, Nunc, Denmark) were coated with the test protein or background control protein for 16 h at +4°C. Samples were diluted into the respective background control and incubated for 1 h (VP2 and NS2) or 1.5 h (NS1 and NS3) at RT. ELISA plates were blocked for 3 h at RT with 2% (wt/vol) BSA in PBS and washed 3 times with PBS, and diluted serum samples were added and incubated for 1.5 h at 37°C. Wells were washed 3 times with PBS-Tween before 45 min (VP2, NS1, and NS3) or 1 h (NS2) of incubation at 37°C with rat anti-mouse IgG1 heavy chain-HRP (MCA336P; AbDSerotec, United Kingdom), followed by 3 washes with PBS-Tween, addition of the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate, and finally addition of hydrogen peroxide stop solution. Absorbance values were measured at 450 nm, corrected optical density (OD) ($COD = OD_{protein} - OD_{background}$) values were calculated, and then titers

were calculated by linear regression to a cutoff based on the COD value of negative-control sera at a 1:50 dilution. Sera that were antibody negative at a 1:50 dilution (the lowest dilution tested) were set to that titer (50; i.e., a 1.7 $\log_{10}$ titer) for calculating means and performing statistical analysis.

Western blotting using pooled sera from mice immunized with $vVP_2NS_{1/2/3}$ was performed to verify the presence of antibodies recognizing recombinant purified VP2 of BTV-8 and NS1 and NS2 of BTV-2. Purified recombinant proteins (2 to 4 μg) and associated background control antigens were diluted in PBS and then run on an SDS-PAGE gel for Western blotting as described above, with diluted sera as the primary antibody and rat anti-mouse heavy chain IgG1-HRP as the secondary antibody.

(iii) Detection of lymphoproliferative responses. Immediately following euthanization, spleens of all animals were removed and individually flushed with sterile PBS, and single-cell suspensions were centrifuged over Ficoll-Paque Plus medium (GE Healthcare, United Kingdom) for 15 min at 1,300 × *g* and +4°C. Isolated mononuclear cells were restimulated, in quadruplicate, with individual proteins and relevant background controls at 0.03 μg of protein per well. Lysate from Sf9 cells or from purified nontransfected *E. coli* BL21-AI (Invitrogen, United Kingdom) was used as background controls for VP2, NS1, and NS3 or for NS2, respectively. The alamarBlue reagent (Invitrogen, United Kingdom) was used to quantify cell proliferation. Briefly, after 5 days of incubation at 37°C and 5% CO_2, 20 μl/well of the alamarBlue reagent was added for 18 h, and then the absorbances at 570 nm and 595 nm were measured by spectrophotometry. OD values (OD = OD at 570 nm [OD_{570}] − OD_{595}) and corrected OD (COD = $OD_{protein}$ − $OD_{background}$) values were calculated for BTV protein-specific stimulations.

(iv) Statistical analysis. Data were analyzed using the statistical program R (46). Due to the structure of the sample data, nonparametric Kruskal-Wallis tests for independent groups were used for analysis among all immunized groups of mice, and Mann-Whitney tests for independent groups were used for analysis between two immunized groups of mice. Statistical significance of the tests was set to a *P* value of ≤0.05 (∗) or ≤0.01 (∗∗) unless otherwise specified, and all values are provided as the indicated means ± standard deviations (SD), where applicable.

Evaluation of bovine BTV-8-specific polyclonal antibody recognition of recombinant VP2, NS1, and NS2. Cattle sera obtained 3 weeks after experimental infection with BTV-8, after two vaccinations with a commercial inactivated vaccine against BTV-8, after eight immunizations with commercial inactivated vaccines against BTV-8, and from noninfected and non-BTV-vaccinated animals (negative controls) were tested for the presence of antibodies to VP2, NS1, NS2, and background controls (Sf9 cell lysate and *E. coli* BL21-AI) by Western blotting, as described above. Diluted sera was used as the primary antibody, and sheep anti-bovine IgG-HRP (AAI23P; AbDSerotec, United Kingdom) was used as the secondary antibody.

RESULTS

Expression and identification of recombinant BTV proteins. Recombinant BTV proteins were successfully produced in Sf9 cells (VP2, VP5, NS1, and NS3) or *E. coli* BL21-AI (NS2). Mass spectrophotometry was used to verify that the expressed and produced proteins were those targeted. Using MALDI-TOF MS analysis, the recombinant protein VP2 was identified with 26% protein sequence coverage as VP2 of BTV-8. The remaining recombinant proteins, VP5, NS1, NS2, and NS3, were identified by LC-Orbitrap MS/MS analyses as VP5 of BTV-8, NS1 of BTV-2, NS2 of Corsican bluetongue virus, and NS3 of BTV, with 46%, 41%, 64%, and 62% protein sequence coverage, respectively.

Degree of purification and quantification of recombinant BTV proteins. Two purification protocols using either nickel (columns) or cobalt (plate wells) affinity were tested for each recombinant protein, and the method chosen was based on percent purity as determined using digital image analysis as well as reproducibility (data not shown). Accordingly, VP2, NS1, and NS2 were purified using cobalt affinity, whereas NS3 and VP5 were purified using nickel affinity. Next, optimized lysis and elution buffers for each recombinant protein (Table 1) were determined by adjustment of salt, pH, or imidazole concentrations, addition of detergents, or changes in temperature (Fig. 1), followed by protein presence and purity verification using Western blotting, digital image analysis of Coomassie staining of SDS-PAGE gels, and consideration of efficiency and reproducibility; representative sample data for NS3 are shown in Fig. 2. Using the optimized protocols detailed in Table 1, recombinant VP2 yielded 95% comparative purity, NS1 yielded 51%, NS2 yielded 85%, and NS3 yielded 77% (Fig. 3). No suitable purification protocol yielding sufficient purity of VP5 was determined (7 to 11% comparative purity; Fig. 3) using the tested methods, and therefore, this recombinant protein was excluded from use in protein-specific immunogenicity studies.

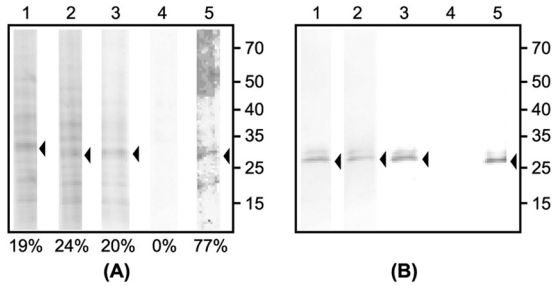

FIG 2 Degree of purification and presence of recombinant His-tagged NS3 of BTV-2. Coomassie-stained SDS-PAGE (A) or Western blot using mouse antihistidine monoclonal antibodies (B) showing the degree of purification and presence of NS3 after purification using His SpinTrap columns with different adjustments of lysis buffer contents: following the manufacturer's protocols (lane 1), adding 2% octyl glucoside (lane 2), increasing the salt concentration 5× (lane 3), decreasing the salt concentration 10× (lane 4), and using NP-40 lysis buffer (lane 5, optimized lysis buffer). Semiquantitive purity percentages are indicated below each lane in panel A. Arrowheads indicate recombinant BTV proteins at expected molecular masses. Molecular mass markers (in kDa) are noted at the right of each image.

Using the optimized protocols, the final concentrations of VP2, NS1, NS2, and NS3 after purification, dialysis, and sterile filtration (VP2 and NS2) were determined to be 49.6 μg/ml, 88.9 μg/ml, 52.8 μg/ml, and 10.7 μg/ml, respectively. NS1 and NS3 were not detected after sterile filtration and therefore were not sterile filtered after dialysis.

Stability of recombinant BTV proteins. Stored purified VP2, NS1, and NS2 were detected at their expected molecular weights by Western blotting using mouse antihistidine monoclonal antibodies at all studied time points and conditions, including at 80 weeks at −80°C, 88 weeks (experiment termination) at +4°C, and in dialyzed and sterile-filtered aliquots (VP2 and NS2), except in the case of purified NS2 after 5 weeks of storage at +4°C (Table 2). The recombinant proteins evaluated at this time point were not purified using optimized protocols, while those evaluated after long-term storage were purified using optimized protocols. Purified VP5 and NS3 were also detected at both storage temperatures at 0, 1, 2, and 5 weeks but only as crude and not purified proteins at +4°C at 88 weeks.

According to image analysis, the percent purity of VP2, NS1,

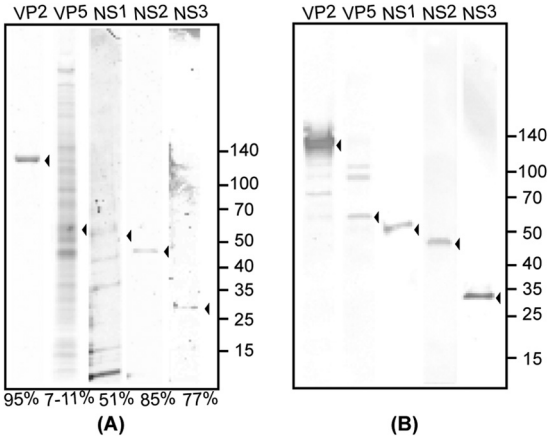

FIG 3 Expression and purification of recombinant VP2 and VP5 from BTV-8 and NS1, NS2, and NS3 from BTV-2. A Coomassie-stained SDS-PAGE gel (A) or Western blot using mouse antihistidine monoclonal antibodies (B) confirming expression and purification of recombinant His-tagged VP2, VP5, NS1, and NS3 from baculovirus-infected Sf9 cells and NS2 from transformed *E. coli* BL21-AI is shown. Recombinant proteins were purified using cobalt or nickel affinity. Semiquantitive purity percentages are indicated below each lane in panel A. Arrowheads indicate recombinant BTV proteins at expected molecular masses. Molecular mass markers (in kDa) are noted at the right of each image.

and NS2 remained approximately stable between 0 and 5 weeks of storage at both +4°C and −80°C, while VP5 and NS3 decreased from 11% and 34% to 3 to 4% and 22 to 23%, respectively, after 1 week of storage at +4°C and −80°C, after which the protein purity then remained approximately stable for at least 4 weeks (Table 2). The low percentages of NS3 purity are due to purification using nonoptimized protocols. No change in the size or number of protein bands, indicating protein degradation, was detected for any protein throughout the course of this study.

Immunogenicity of recombinant BTV proteins in mice. (i) Humoral responses. Specific serum IgG1 antibodies induced by VP2 in mice immunized with either vVP_2 or $vVP_2NS_{1/2/3}$ were detected in sera obtained at day 42, 2 weeks after the second immunization (mean titer, 2.3 ± 0.6 $\log_{10}$ or 2.3 ± 0.4 $\log_{10}$, respectively; $P \leq 0.05$ or $P \leq 0.01$ compared to results for controls, respectively) but not after the first immunization at day 28 (Fig. 4). Although levels of VP2-specific antibody titers increased between day 28 and day 42 (mean titers, 1.8 ± 0.1 $\log_{10}$ and 2.0 ± 0.4 $\log_{10}$, respectively) in sera of mice immunized with $vNS_{1/2/3}$, which were not immunized with VP2, this difference was not statistically significant ($P = 0.09$).

NS2 induced specific serum IgG1 antibodies (BTV-2), as detected by Western blotting and indirect ELISA, in mice immunized with $vNS_{1/2/3}$ (mean titer, 2.48 ± 0.65 $\log_{10}$; $P \leq 0.01$) or $vVP_2NS_{1/2/3}$ (mean titer, 2.08 ± 0.35 $\log_{10}$; $P \leq 0.05$), which were significant compared to those in controls or in mice immunized with vVP_2 (mean titer for both groups, <1.7 ± 0 $\log_{10}$) at day 42 (Fig. 4). NS2-specific antibodies were also detected at day 28 in mice immunized with $vNS_{1/2/3}$ (mean titers, 2.19 ± 0.58 $\log_{10}$; $P \leq 0.05$) but not at significant titers compared to those for controls in mice immunized with $vVP_2NS_{1/2/3}$ (mean titer, 2.03 ± 0.38 $\log_{10}$; $P = 0.058$). Although NS2-specific serum antibody titers were higher when induced by $vNS_{1/2/3}$ than when induced by $vVP_2NS_{1/2/3}$ on day 28 or on day 42, this difference was not statistically significant ($P = 0.739$ and $P = 0.259$, respectively).

NS1-specific IgG1 antibodies were not detected in mice from any group in sera from day 28, and further analyses were limited due to low obtained serum sample volumes on day 42 (data not shown). Due to the limited quantities of pure NS3 protein produced, a reliable assay to verify NS3-specific IgG1 antibody titers in mouse sera could not be adequately developed (data not shown).

(ii) Cellular responses. Protein-specific lymphocyte proliferative responses to VP2, NS1, and NS2 (Fig. 5) but not to NS3 (data not shown) were detected *ex vivo* following restimulation with individual proteins and corresponding background controls. VP2-specific proliferative lymphocyte responses were detected in mice immunized with vVP_2 (mean COD, 0.231 ± 0.012) or with $vVP_2NS_{1/2/3}$ (mean COD, 0.152 ± 0.121), which were significant compared to those for controls (mean COD, 0.007 ± 0.017; $P \leq 0.01$ and $P \leq 0.05$, respectively). NS1-specific proliferation of lymphocytes was also significant compared to results for controls (mean COD, 0.020 ± 0.032) in mice immunized with $vNS_{1/2/3}$ (mean COD, 0.162 ± 0.082; $P \leq 0.01$) and $vVP_2NS_{1/2/3}$ (mean COD, 0.159 ± 0.109; $P \leq 0.05$). Although COD values representing NS2-specific lymphocyte proliferative responses were low for mice immunized with $vNS_{1/2/3}$ (mean COD, 0.054 ± 0.031) and $vVP_2NS_{1/2/3}$ (mean COD, 0.063 ± 0.031), responses of those immunized with $vVP_2NS_{1/2/3}$ were significant compared to those for controls (mean COD, 0.017 ± 0.030; $P \leq 0.05$).

Recognition of recombinant BTV proteins by BTV-specific bovine polyclonal antibodies. Bovine serum IgG antibodies rec-

TABLE 2 Detection and percentages of purity of recombinant BTV proteins as determined by Coomassie staining of SDS-PAGE gels and Western blotting at indicated storage week, condition, and temperature[a]

	Detection of protein																				
			1 wk (purified)				2 wks (purified)				5 wks (purified)										
	0 wks (purified)		+4°C		−80°C		+4°C		−80°C		+4°C		−80°C		30 wks (−80°C, WB)			80 wks (purified, −80°C)		88 wks (WB, +4°C)	
Protein	% pur.	WB	% pur.	WB	% pur.	WB	% pur.	WB	% pur.	WB	% pur.	WB	% pur.	WB	Purified	Dialyzed	Filtered	% pur.	WB	Crude	Purified
VP2	94	+	99	+	98	+	93	+	97	+	96	+	94	+	+	+	+	90	+	+	+
VP5	11	+	4	+	3	+	6	+	6	+	3	+	3	+						+	−
NS1	51	+	43	+	51	+	43	+	*	+	41	+	45	+	+			39	+	+	+
NS2	72	+	*[b]	+	57	+	75	+	50	+	0	−	81	+	+	+	+	83	+	+	+
NS3	34	+	23	+	22	+	27	+	23	+	20	+	20	+						+	−

[a] Detection by Coomassie staining of SDS-PAGE gels is given by semiquantitative purity percentages (% pur.), whereas detection by Western blotting (WB) is indicated as present (+) or not present (−). Blank cells, analyses were not performed.

[b] *, results were unreliable due to poor image quality.

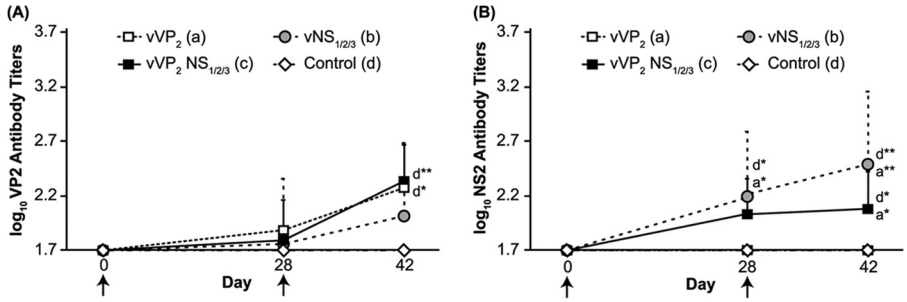

FIG 4 Kinetics of protein-specific serum antibody titers directed against VP2 of BTV-8 (A) or NS2 of BTV-2 (B) in immunized mice. Mice (represented by group means of $\log_{10}$-transformed values) were immunized twice at a 4-week interval with one of three combinations of purified recombinant BTV proteins and AbISCO-100 (abbreviated as vVP2 [$n = 5$], vNS1/2/3 [$n = 6$], or vVP2NS1/2/3 [$n = 6$]) or with AbISCO-100 alone (Control; $n = 6$). Sera were collected 4 and 2 weeks after the first and second vaccinations (indicated by arrows), respectively, and analyzed by indirect ELISA. Lines between data points are included for illustrative purposes. Standard deviations are shown by upward deflection lines. Statistical significance is indicated by asterisks ($P \leq 0.05$ [*] or $P \leq 0.01$ [**]) and the corresponding group names.

ognized purified recombinant VP2 (BTV-8) and NS2 (BTV-2) but not NS1 (BTV-2) (data not shown), as determined by Western blotting using polyclonal sera from cattle immunized with vaccines based on inactivated BTV-8 and from experimentally BTV-8-infected cattle (Fig. 6). In all cases, no bands were detected by Western blotting using the same sera to blot against background controls or using negative-control sera (data not shown).

DISCUSSION

In this article, we present the rational design and systematic development process behind the formulation of a DIVA subunit vaccine against BTV. We expressed and produced the recombinant BTV proteins VP2, VP5, NS1, NS2, and NS3 and showed that all five proteins can be stored for practical vaccine use, that VP2, NS1, NS2, and to some extent NS3 can be purified for use in a subunit vaccine, and that VP2, NS1, and NS2 induced humoral and/or cellular immune responses in mice. Furthermore, we showed that VP2 and NS2 were recognized by antibodies produced following BTV-8 infection. Based on these data, which were confirmed in a subsequent study with analyses indicating high immunogenicity in cattle (47), the combination of VP2, NS1, and NS2 associated with an ISCOM-Matrix adjuvant may compose a promising subunit vaccine candidate against BTV-8 for ruminants. The rational design of this experimental vaccine enables DIVA and allows for adaptation to new or additional BTV serotypes.

Proteins were initially selected to induce protection against BTV through serotype-specific neutralizing antibody responses (VP2 and VP5 of BTV-8) (28, 40) and cross-serotype T cell responses (NS1, NS2, and NS3 of BTV-2). The structural proteins were selected to target BTV-8 because of recent European outbreaks that caused birth malformations and other clinical signs in cattle (4). The inclusion of nonstructural proteins from a different serotype was based on the availability of production systems for these proteins in our laboratories and on evidence of the proteins' genetic conservation across serotypes (48), which suggested they could compose the foundation of a versatile vaccine (25). That is, this vaccine candidate may later be adapted to other or multiple BTV serotypes by exchanging or including VP2 originating from those target serotypes. Furthermore, by excluding the BTV protein VP7, this experimental vaccine enables DIVA by monitoring VP7-specific antibodies using well-established commercial assays detecting BTV infections of any serotype.

Baculovirus (VP2, VP5, NS1, and NS3) and *E. coli* (NS2) expression systems were utilized during this study. Both systems have been successfully used in BTV recombinant protein production before (16, 35, 49) and are practical for vaccine application because of their availability, affordability, and potential capability to produce large quantities of functional protein (50). In addition to the subunit vaccine design presented here, other approaches, including reverse genetics technologies, are also increasingly practical for BTV vaccine designs, since they demonstrate DIVA potential (51) and show promising early success against multiple serotypes (52). However, while the systems employed in this study can be scaled up from laboratory to industrial settings, the flexibility of subunit vaccine design also allows for proteins to be pro-

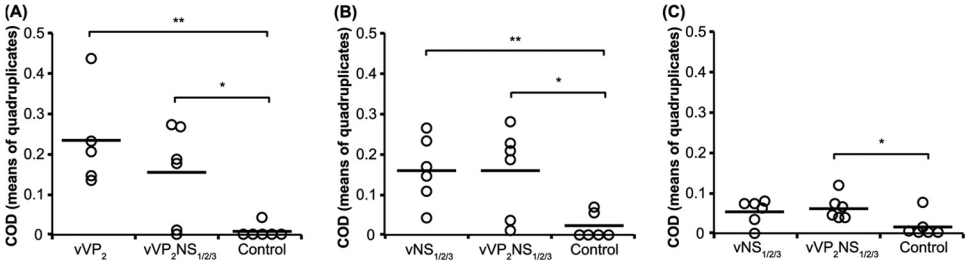

FIG 5 Proliferative responses of spleen lymphocytes of immunized mice stimulated *ex vivo* with VP2 of BTV-8 (A), NS1 of BTV-2 (B), or NS2 of BTV-2 (C). Mice were immunized as indicated in Fig. 4. Proliferation is expressed as corrected OD values (mean of quadruplicates) after 5 days of stimulation with VP2, NS1, NS2, or control antigen and addition of the alamarBlue reagent. Dots represent individual mice, and horizontal lines represent group means. Statistical significance is indicated by asterisks ($P \leq 0.05$ [*] or $P \leq 0.01$ [**]) and horizontal brackets between corresponding groups.

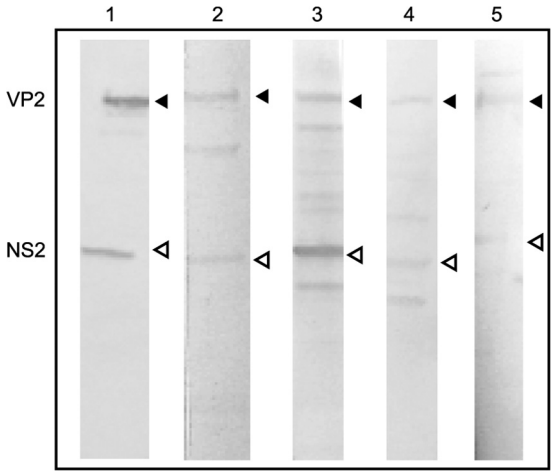

FIG 6 Western blot indicating specific recognition of purified recombinant VP2 of BTV-8 and NS2 of BTV-2 by serum antibodies induced by BTV-8 vaccination or infection. Western blots using mouse anti-histidine tag monoclonal antibodies (lane 1), murine sera after two immunizations with vVP2NS1/2/3 (lane 2), and bovine serum after two immunizations with a commercial inactivated vaccine against BTV-8 (lane 3), eight immunizations with commercial inactivated vaccines against BTV-8 (lane 4), and 3 weeks after experimental infection with BTV-8 (lane 5) are shown. Solid arrowheads indicate VP2 (111 kDa), and white arrowheads indicate NS2 (40 kDa).

duced in newer systems that might facilitate even greater and more cost-effective production, such as baculovirus and silkworm larvae rather than insect cells (53).

The addition of a polyhistidine tag to each recombinant protein enabled affinity purification using convenient commercial methods. By adjusting the lysis buffer, purification method, and elution buffer, we were able to purify VP2, NS1, NS2, and to some extent NS3 but not VP5. VP2 and VP5 are the BTV outer capsid proteins and form trimers that are triskelion and globular in structure, respectively (54, 55). While VP2 mediates cell entry by attaching to host cell receptors and facilitating receptor-mediated endocytosis (56), VP5 has a hydrophobic membrane-inserting domain and exhibits membrane-permeabilizing capability (55). Although our VP2 was solubilized using salt concentrations recommended by the manufacturer, we were unable to reproducibly purify VP5, perhaps due to failure to solubilize the protein despite adjustments to salt and pH concentrations, addition of detergents, and changes in temperature.

The identities of our recombinant proteins were verified using peptide sequencing before proceeding to evaluate immunogenicity in animal experiments. Using different experimental formulations for mouse immunizations allowed for comparisons of protein-specific immune responses, including assessment of suppressive or synergistic effects of individual proteins, with a minimum number of animals ($n = 6$ per group). Of the proteins evaluated, the induction of humoral (VP2 and NS2) or cell-mediated (VP2, NS1, and NS2) immunogenicity was detected for all proteins except NS3. NS3 was the most difficult to purify for immunization and was used at the lowest concentration due to its insufficient production. As a consequence, we were unable to develop a reliable assay to detect NS3-specific antibodies. Above all, no significant differences in NS3-specific lymphocyte proliferation were observed among immunized mice and controls. Taking these things together, we decided to exclude our recombinant NS3 protein from further analyses.

Protein-specific humoral analyses of VP2 and NS2 detected specific serum antibodies to both proteins in immunized mice. It is accepted that serotype-specific immunity induced by VP2 is required for full protection against BTV infection and is mediated by neutralizing antibody induction (17, 18). When it was later evaluated in a subsequent study in cattle, it was shown that the purified recombinant VP2 protein presented herein induced strong BTV-8-neutralizing antibody titers (47). In both studies, only NS2-specific antibodies were detected after just one immunization. Furthermore, NS2 also tended to induce stronger antibody responses in this study when administered with nonstructural proteins alone ($vNS_{1/2/3}$) compared to administration with VP2 ($vVP_2NS_{1/2/3}$), though this difference was not statistically significant. The protective role of NS2-specific antibodies is not clear, but it is possible that mechanisms such as phagocyte opsonization and complement activation may be mediated by nonneutralizing antibodies. However, the role of antibodies specific to internal virus proteins in such processes remains to be clarified.

We did not detect NS1-specific antibody induction after the first vaccination and were unable to determine their presence after two immunizations due to limited serum quantities. In cattle, however, this recombinant purified NS1 protein induced strong specific serum antibody responses after two vaccinations (47). We believe that the difference observed between these two studies may be due to species differences between cattle and mice or to differences in assay sensitivities.

In contrast, restimulation with NS1, as well as VP2 and NS2, did induce specific lymphocyte proliferation in immunized mice. However, whereas NS1 also induced lymphocyte proliferation in cattle, VP2 and NS2 did not (47). This may be due to species differences in protein-specific immune responses, to lower sensitivity of the bovine assay than of the murine assay, or to differences in lymphocyte origin (spleen lymphocytes for mice; peripheral blood mononuclear cells for cattle). It has been speculated that the induction of T cells may contribute to multiserotype protection against BTV, particularly when induced by the nonstructural proteins (25). Therefore, we chose AbISCO-100, an ISCOM-Matrix, as an adjuvant. ISCOM-Matrices are understood to function through several mechanisms that help stimulate $CD8^+$ T cells in particular but also $CD4^+$ T cells, leading to high antigen-specific antibody production. This is mediated by efficient and prolonged uptake of associated antigens by antigen-presenting cells, by facilitating quick entry into the draining lymphatic system, by supporting antigen transport and availability to class I and II major histocompatibility complex pathways, by inducing relevant cytokine production, and by presenting antigens as particulates that mimic native virus (57–60). Here, the specific lymphocyte proliferative responses support previous research demonstrating the induction of cellular immunity following immunization with proteins or virus combined with ISCOM-based adjuvants (61–63).

After determining protein-specific immunogenicity in mice, we used *in vitro* tests to confirm that the proteins which induced humoral immune responses in mice (VP2 and NS2) were specifically recognized by serum antibodies of BTV-8-vaccinated cattle (47) and to demonstrate for the first time that both proteins were also specifically recognized by serum antibodies of BTV-8-infected cattle. We did not detect NS1-specific antibodies in immunized mice, and although such antibodies may be induced by BTV

infection (41, 64), they are only rarely detected following immunization with inactivated BTV vaccines. The poor recognition of NS1-specific antibodies by Western blotting using sera from infected cattle in this study may be due to conformational differences in B cell epitopes between our recombinant NS1 and the native viral protein or to a lower sensitivity of our assay than of previously used methods. Unfortunately, we were unable to determine whether the T cell epitopes of our recombinant proteins were recognized by memory T lymphocytes induced by BTV-8 infection, since we did not have access to lymphocytes from such animals. In the case of NS2 (BTV-2), the BTV-8-specific antibody recognition was particularly encouraging because it indicated NS2 cross-reactivity between serotypes. This supports suggestions that the nonstructural proteins may provide protection against multiple, or novel, BTV serotypes (25).

In conclusion, we began with five immunologically relevant BTV proteins that were produced in scalable expression systems and followed systematic steps of protein-specific identification, stability assessment, purification method optimizations, and immunogenicity determinations with a minimum number of mice, to ultimately develop an experimental vaccine composed of the stable and purified recombinant BTV proteins VP2, NS1, and NS2 and an ISCOM-Matrix adjuvant. Since this novel vaccine induced both humoral and cell-mediated immune responses in mice and additionally was recognized in part by BTV-specific bovine antibodies, we believe it may provide protection against BTV-8 infection in cattle. Furthermore, by using rational vaccine design, this recombinant subunit vaccine may be adapted to additional or multiple serotypes of BTV depending on local epidemiology while still maintaining DIVA compliancy.

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